Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Comparison of *heat shock protein 70 kDa* and *18S rDNA* genes for molecular detection and phylogenetic analysis of *Babesia vogeli* from whole blood of naturally infected dogs

Patrícia G. Paulino[a], Marcus S. Pires[b], Claudia B. da Silva[a], Maristela Peckle[b], Renata L. da Costa[b], Gabriela L.V. Vitari[a], Ana Paula M. de Abreu[b], Nádia R.P. Almosny[c], Carlos L. Massard[b], Huarrisson A. Santos[a,*]

[a] Department of Epidemiology and Public Health Veterinary Institute, Federal Rural University of Rio de Janeiro, Rio de Janeiro, Brazil
[b] Department of Animal Parasitology, Veterinary Institute, Federal Rural University of Rio de Janeiro, Rio de Janeiro, Brazil
[c] Departament of Veterinary Clinic and Pathology, Federal Fluminense University, Niteroi, Brazil

ARTICLE INFO

Keywords:
Babesiosis
Real-time polymerase chain reaction
Heat shock protein 70 kDa
Dogs

ABSTRACT

A total of 300 blood samples of domiciliated dogs in rural and urban areas of southeast Rio de Janeiro State, Brazil, were used to compare the *18S ribosomal DNA* region (*18S rDNA*) and the *heat shock protein 70 kDa (hsp70)* gene for molecular detection of *Babesia vogeli* and to perform a phylogenetic study comparing the two genes for *B. vogeli* classification. Using conventional polymerase chain reaction (cPCR) of *18S rDNA* and *hsp70* sequences, we were able to detect *B. vogeli* with the same sensitivity (96.15%) and specificity (99.63%). However, sequencing revealed one false positive (*Rangelia* sp.) for *18S rDNA* that was not detected by *hsp70*. This is the first report of an organism closely related to the *Rangelia vitalii* parasite of dogs in Brazil. In the *hsp70*-cPCR and *hsp70*-qPCR comparison, 15.66% of samples were considered positive by quantitative (q)PCR, significantly more than was detected by cPCR (8.66%). In addition to the high conservation of the *18S rDNA*, phylogenetic analysis showed that the *hsp70* gene can be used to describe phylogenetic relationships between canine piroplasmids with more accuracy than *18S rDNA*. According to these findings, the qPCR method has greater sensitivity than cPCR for detection of *B. vogeli* in naturally infected dogs. The *hsp70*-qPCR detection limit was 10 copies, with an efficiency of 100.30% and a determination coefficient (R^2) of 0.998. The development of this qPCR method provides a highly sensitive approach for *B. vogeli* molecular detection and a tool that is capable of quantifying parasitemia levels in whole blood samples from dogs. The primers and probes were designed to be specific for *B. vogeli*, though analytical specificity of the assay has not been tested *in vitro* with DNA of certain *Babesia* species that infect dogs. The *hsp70* gene is a precise molecular marker for *Babesia* phylogeny, especially species that infect dogs.

1. Introduction

Canine babesiosis, a disease with a wide geographical distribution (Piana and Galli-Valerio, 1895), is also known as piroplasmosis (Cassini et al., 2009). The causative agents are responsible for different degrees of pathogenicity in their hosts, tending to present high mortality for animals when introduced in endemic areas, as well as for animals that are stressed due to other diseases, immunosuppressed by steroids or splenectomized (Solano-Gallego et al., 2016).

The most common diagnostic methods for canine babesiosis include the direct identification of merozoites inside erythrocytes or free in blood smears, serological methods for detecting antibodies, and DNA amplification by polymerase chain reaction (PCR) (Solano-Gallego et al., 2016). Molecular methods allow for the direct detection of hemoparasite DNA in biological samples with increased specificity, even under conditions of very low parasitemia, a condition that is characteristic of asymptomatic carriers or chronically infected animals (Freeman, 2012). In addition, such approaches enable distinguishing between different parasite species (Carret et al., 1999). Similarly, molecular techniques have revolutionized the field of parasitic disease diagnostics. Several studies have been conducted to compare the most common techniques to pathogen detection, with many reporting that molecular tools are more reliable than other methods (Otranto et al., 2011; Kubelová et al., 2013; Mrljak et al., 2017). A variety of molecular

* Corresponding author.
E-mail addresses: huarrisson@yahoo.com.br, huarrisson@yahoo.com.br (H.A. Santos).

https://doi.org/10.1016/j.ttbdis.2018.01.013
Received 5 August 2017; Received in revised form 11 January 2018; Accepted 24 January 2018

techniques are available in addition to conventional PCR (cPCR), such as loop-mediated isothermal amplification assay (LAMP) (Müller et al., 2010), quantitative PCR (qPCR) (Vilela et al., 2013), restriction-fragment length polymorphism-PCR (RFLP-PCR) (Carret et al., 1999), and nested PCR (nPCR) (Ano et al., 2001). qPCR offers several advantages, including the possibility of monitoring every cycle of the reaction in real time (Heid et al., 1996), and it is performed in a closed-tube system, eliminating post-PCR manipulation, minimizing contamination risk and resulting in a much faster assay (Whitcombe et al., 1999). Another great difference is the possibility of quantifying the DNA targets (Heid et al., 1996). A standard curve generated by serial dilution of the target may be applied to determine the copy number for any unknown sample (Bustin et al., 2009). Digital PCR comprises another modality of PCR capable of quantifying targets; however, qPCR is the only method available to date that has been assessed for *B. vogeli* target quantification.

Despite all these beneficial characteristics, success relies on the design of primers targeting suitable molecular markers (Lymbery and Thompson, 2012). The most common target used to diagnostic babesiosis worldwide is the *18S ribosomal DNA* (*rDNA*) gene (Skotarczak, 2008). Ribosomal DNA is highly conserved, and its sequences are very useful for routine detection of genes, but it is important to emphasize the possibility that species may not be recognized with recently occurring evolutionary divergence (Lymbery and Thompson, 2012). Considering the difficulty of species differentiation using 18S *rDNA*, other targets may be used as an alternative, including *cytoplasmic protein 29 kDa*, *thrombospondin-related adhesive protein* and *heat shock protein 70 kDa* (*hsp70*) genes (Fukumoto et al., 2003; Zhou et al., 2006; Yamasaki et al., 2007). The *hsp70* gene is also highly conserved and presents more interspecific variability when compared to *18S rDNA* in phylogenetic studies (Yamasaki et al., 2007). Application of this target is also recommended for taxonomic classification of *Babesia* and *Theileria* species (Yamasaki et al., 2007). However, only a few sequences of the *hsp70* gene from piroplasm species from different parts of the world are available, which may limit phylogenetic analyses of this group of parasites.

This study aimed to standardize and compare the performance of qPCR with a cPCR technique targeting *hsp70* of *B. vogeli* for the diagnosis of this agent in naturally infected dogs. To this end, the *hsp70* gene and the *18S rDNA* molecular markers were compared, and a preliminary phylogenetic study comparing the *hsp70* gene with the *18S ribosomal DNA* sequence was performed.

2. Materials and methods

2.1. Sampling procedures

A total of 300 blood samples were collected from domestic dogs domiciliated in rural and urban areas of municipalities of Seropédica, Itaguaí, Mangaratiba and metropolitan region of Rio de Janeiro, during the years 2016 and 2017. During sample collection, it was possible to observe the presence of ticks on the dogs and in the environment. *Rhipicephalus sanguineus* sensu lato was the main tick species present, though some *Amblyomma ovale* specimens were identified as well.

A sample of blood was drawn from each animal by cephalic venipuncture, placed in sterile tubes containing ethylenediaminetetraacetic acid and stored at −80 °C until DNA extraction.

2.2. DNA extraction

DNA was extracted from 300 µL of each blood sample using the **Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA)** according to the manufacturer's recommendations. After extraction, the DNA samples were quantified using a Spectrophotometer **Nanodrop ND-2000® (Thermo Fisher Scientific, Wilmington, DE, USA)**, diluted and stored in aliquots of 100 ng/µL.

2.3. Standard sample

The standard sample was obtained from a symptomatic dog with intraerythrocytic inclusions of *B. vogeli* observed by microscopic examination. cPCR was performed targeting the *18S rDNA* region of *B. vogeli* (Carret et al., 1999). The fragments obtained were purified with Wizard® SV Genomic DNA Purification System **(Promega, Madison, WI, USA)** and sequenced by the Sanger method (Sanger et al., 1977). The sequences were analyzed using DNA Sequence Assembler® 4.0 (Heracle BioSoft, www.DnaBaser.com), revealing 100% identity with *B. vogeli*. The *18S rDNA* partial sequence was deposited in the GenBank database under the accession number MF459002. This sample was quantified in the *hsp70*-qPCR method, with a Cq value of 29.81 cycles (≅100 copies). This positive control was used in all assays.

2.4. Standardization of qPCR

Oligonucleotides were designed using Primer Express® 3.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA) based on sequence alignment of the *hsp70* gene from *B. vogeli* and other piroplasms available in GenBank. The characteristics of the primers and the probe were evaluated using Oligo Analyzer (Gene Link™, Hawthorne, NY, USA). Primer and probe specificity was tested *in silico* using primerBLAST (NCBI, Bethesda DM, USA) and FastPCR software (Kalendar et al., 2017), respectively. The primers were designed to amplify a fragment of 118 bp of the *B. vogeli hsp70* gene, as follows: *BvqF* (5′-GCTGGTGACACCCAC CTT-3‘) and *BvqR* (5‘- GGCACGCTTGTTGGTC −3′). The TaqMan® hydrolysis probe was named Bvq (5′-*NED*-CCTCCTTGTTGAGCACT-*MGB*-3′). The oligonucleotides were synthesized by Invitrogen (Thermo Fisher Scientific Inc., Waltham, MA, USA). The predicted annealing temperature of the primers was 60 °C, and that of the probe was 72 °C.

Primer and probe concentrations were analyzed using concentration curves generated from the standard sample DNA as a template. The optimal primer concentration was determined considering the minimum concentration necessary to achieve the lowest cycle quantification (Cq) and maximum normalized report fluorescence signal (ΔRn) in the absence of non-specific peaks by melting curve analysis. The qPCR reactions were performed using DNA intercalating reagents, and the standard sample was used as a positive control. PCR was performed in triplicate for each condition established for primer testing (200 nM/200 nM, 200 nM/400 nM, 200 nM/600 nM, 200 nM/800 nM, 400 nM/200 nM, 400 nM/400 nM, 400 nM/600 nM, 400 nM/800 nM, 600 nM/200 nM, 600 nM/600 nM, 600 nM/800 nM, 800 nM/200 nM, 800 nM/600 nM and 800 nM/800 nM) according to the commercial protocol. The reactions were carried out in a total volume of 12 µL containing 1X Syber Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific Inc., Waltham, MA, USA) and 3 µL (100 copies) of *B. vogeli* DNA using the following the thermocycling conditions: 95 °C for 10 min and 40 cycles of 95 °C for 20 s, and 60 °C for 1 min. A melting curve was generated at the end of the thermocycling to verify the specificity of the amplifications and the formation of primer dimers.

The optimal probe concentration was optimized in triplicate for each concentration (50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, and 350 nM). The reaction was performed in a final volume of 12 µL containing 1X commercial TaqMan Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific Inc., Waltham, MA, USA), 800 nM each primer, and 3 µL (100 copies) *B. vogeli* DNA. The optimal probe concentration for qPCR was based on the result of the lowest Cq and the maximum ΔRn obtained in the target detection assay.

To avoid contamination during DNA extraction and preparation of PCR mixture, these steps were performed in separate rooms, and filter tips were used in all steps.

2.5. Standardization of hsp70 gene cPCR

Primers for cPCR were designed to amplify a fragment of 523 bp of